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Crystallization and preliminary crystallographic analysis of human glycosylated haemoglobin

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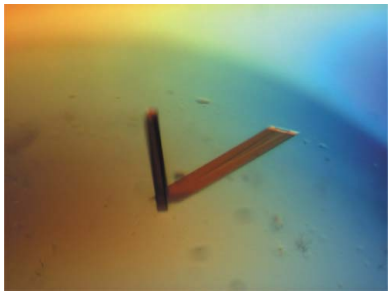

Human glycosylated haemoglobin A_{1C} is a stable minor variant formed *in vivo* by post-translational modification of the main form of haemoglobin by glucose. Crystals of oxyHbA_{1C} were obtained using the hanging-drop vapour-diffusion method and PEG as precipitant. The diffraction pattern of the crystal extends to a resolution of 2.3 Å at 120 K. The crystals belong to space group $C2$, with unit-cell parameters a = 237.98, b = 59.27, c = 137.02 Å, α = 90.00, β = 125.40, $\gamma = 90.00^\circ$. The presence of two and a half molecules per asymmetric unit gives a crystal volume per protein weight (V_M) of 9.70 Å^3 Da^{-1} and a solvent content of 49%.

1. Introduction

The nonenzymatic glycosylation reaction is a common post-translational modification which occurs with a variety of proteins, such as collagen, albumin, tubulin and many others (Robins & Bailey, 1972; Guthrow *et al.*, 1979; Mikšík & Deyl, 1997; Williams *et al.*, 1982). Glucose reacts in a nonenzymatic way with α- and/or ε-amino groups of proteins *in vitro* and *in vivo* (Mikšík & Deyl, 1997; Shapiro *et al.*, 1980). An *in vivo* occurrence of this reaction was confirmed with the discovery of a naturally existing minor human haemoglobin component, HbA_{1C}, which is the product of a slow condensation reaction resulting from the aldehyde properties of the glucose molecule (Cohen & Wu, 1994).

Measurement of HbA_{1C} in blood has been used for more than 20 y for long-term monitoring of the glycaemic status of patients with both type 1 and type 2 diabetes mellitus (Bunn *et al.*, 1975; Baynes *et al.*, 1984; Oltman *et al.*, 1997). However, glycosylated Hb *per se* is an unlikely factor in the pathophysiology of diabetic complications (Zhang *et al.*, 2001).

Haemoglobin A_{1C} (HbA_{1C}) is a stable minor haemoglobin (Hb) variant formed *in vivo* by post-translational modification of the main HbA_1 form with glucose. Originally, HbA_{1C} was identified by cation-exchange column chromatography as it has distinct charge differences compared with unmodified HbA_1 and other glycohaemoglobins (Bunn *et al.*, 1979). Early structural studies of glycohaemoglobins, in particular the HbA_{1C} fraction, demonstrated that glycosylation occurs mainly at the N-terminal amino-acid residues of the haemoglobin β-chains (Bunn *et al.*, 1975). This reaction has been shown to proceed through the initial reversible condensation of glucose and the α-amino groups of Val1 residues of the β-chains, followed by the formation of a Schiff base or aldimine which then undergoes a nearly irreversible intermolecular Amadori rearrangement (Bunn *et al.*, 1975; Koenig *et al.*, 1977) to a more stable ketoamine form, amino-1-deoxyfructose.

However, in view of the numerous glycosylation sites of haemoglobin, the issue of potential double glycosylation of both β-chains, the presence of glycosylated N-terminal valines in the α-chains or an additional glycosylation at any lysine of either the α- or β-chains remains an open question (Peterson *et al.*, 1998). These glycosylated haemoglobins are characterized by similar physical chemical properties and as a result can coelute with HbA_{1C}.

In addition to the importance of the development and standardization of HbA_{1C} quantification methods used in clinical assays, it should be noted that this minor haemoglobin form is an interesting object in itself. For example, it has been shown that the carbohydrate

modification of haemoglobin is the reason for the steric barrier increase to the interaction of allosteric effectors with HbA_{1C} (DeRosa *et al.*, 1998; Syakhovich *et al.*, 2004) and affects its transport function through redistribution of long-lived and short-lived components in photo-dissociated oxygen recombination kinetics (Okenchi *et al.*, 2001; Bokut *et al.*, 2003).

Thus, the study of the glycosylated haemoglobin structure will play an important role in investigations of the pathogenesis of diabetes mellitus and its complications. In the present study, the crystallization and preliminary crystallographic analysis of the haemoglobin HbA_{1C} chromatographic fraction are reported.

2. Materials and methods

2.1. Purification of oxyhaemoglobin A_{1C}

Fresh blood samples from healthy adult volunteers were collected in heparin-containing tubes. After centrifugation of the samples at 3000*g* for 7 min to remove plasma, erythrocytes were washed four times with isotonic NaCl solution (155 m*M*) and centrifuged at 3000*g* for 7 min. Haemolysate was prepared by incubating the cells with cold water. To remove cell debris, the mixture was centrifuged at 277 K for 45 min at 14 000*g*.

After lysis of erythrocytes, haemoglobin HbA_1 was isolated and purified to homogeneity by ion-exchange chromatography on a DEAE Sepharose column (5 × 30 cm; Huisman & Dozy, 1962, 1965). Prior to chromatography, the haemoglobin solution was dialyzed against 0.05 *M* Tris–HCl buffer pH 8.6. The clear red solution was applied onto a DEAE Sepharose column pre-equilibrated with 0.05 *M* Tris–HCl buffer pH 8.5. The protein was then eluted with a reverse linear pH gradient of 0.05 *M* Tris–HCl buffer at pH values ranging from 8.5 to 7.0. This method allows isolation of the homogeneous adult haemoglobin (HbA_1 and minor HbA_2 forms) as well as efficient elimination of organic phosphates from the haemoglobin preparations (Huisman & Dozy, 1965). All chromatographic steps were performed on a BioLogic work station (BioRad, USA).

Fractions of purified HbA_1 were combined, concentrated and non-enzymatically glycosylated *in vitro* at 277 K for 72 h by incubating 5 m*M* HbA_1 in 200 m*M* potassium phosphate buffer pH 7.4 (Watkins *et al.*, 1987) containing glucose in a ratio of 1:100 (haemoglobin: glucose).

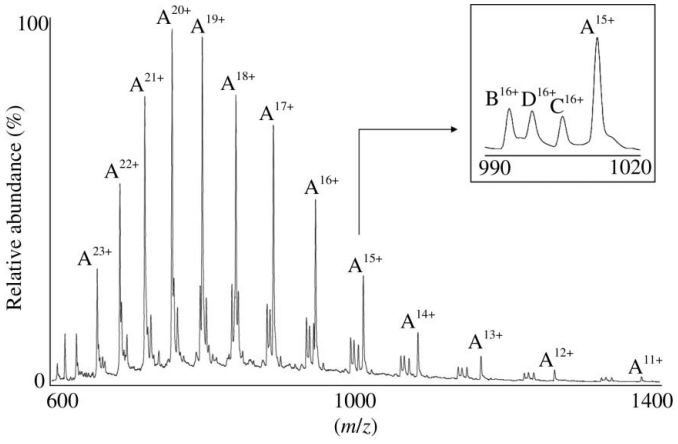

Figure 1
Mass-spectrometric analysis of dissolved crystals. Four species were observed: A, 15 126 Da, assigned to the α-subunit; B, 15 868 Da, assigned to the β-subunit; C, 16 028 Da, assigned to the β-subunit modified by one glucose; D, 15 936 Da, assigned to the α-subunit modified by one glucose with one oxygenated haem.

HbA_{1C} was isolated and purified to homogeneity by cation-exchange chromatography on a CM Sepharose Fast Flow column. The column (2.6 × 30 cm) was pre-equilibrated with 50 m*M* potassium phosphate buffer pH 6.6. Prior to loading, haemoglobin was dialyzed twice against 300 volumes of 50 m*M* potassium phosphate buffer pH 6.6 for 24 h. HbA_{1C} was eluted with a linear gradient of NaCl (0.01–0.05 *M*) in 50 m*M* potassium phosphate buffer pH 6.6 with additional elution with 50 m*M* potassium phosphate buffer pH 6.6 containing 0.05 *M* NaCl. Subsequent washing of the column with a linear gradient of NaCl (0.05–0.1 *M*) in 50 m*M* potassium phosphate buffer pH 6.6 resulted in the elution of non-modified oxyhaemoglobin A_1 and methaemoglobin (MetHb).

The fractions containing HbA_{1C} were combined, concentrated and reloaded onto a CM Sepharose Fast Flow column. Prior to loading, the labile glycohaemoglobin fraction (pre-HbA_{1C}) was eliminated by incubation of the HbA_{1C} preparation at 310 K for 45 min with 20 volumes of sodium acetate buffer pH 5.5 containing 0.1 m*M* NaCl (Antonini & Brunori, 1971).

The final HbA_{1C} preparation was checked by electrophoresis. The haemoglobin samples (4 µl) were analyzed on agarose gel (Hydragel Hb Glyco 'Sebia', France), which separates major haemoglobin A_1 and its glycosylated species. The migration was performed for 30 min at 50 V, stained with a specific dye from the Hydragel Hb Glyco Kit and washed with distilled water. The gel shows that only glycosylated haemoglobin species are present in the purified sample.

The resulting preparations were concentrated by centrifugation using Amicon YM-05 ultrafiltration membranes (USA).

2.2. Mass-spectrometric analysis of haemoglobin prior and after crystallization

Mass-spectrometric studies (Fig. 1) were performed in the positive-ion mode using a commercially available Micromass LC-T electrospray ionization orthogonal time-of-flight mass spectrometer. Calibration was performed using protonated horse heart myoglobin (Sigma).

Crystals were washed three times in drops of 8 *M* ammonium acetate and dissolved in 10 µl 50 m*M* ammonium acetate.

Samples were diluted in a 1:1(*v*:*v*) water–acetonitrile mixture acidified with 1%(*v*/*v*) formic acid to achieve a concentration of 5 pmol μl^{-1}.

Sample solutions were introduced into the mass-spectrometer source with a syringe pump (Harvard Type 55 1111, Harvard Apparatus, South Natick, MA, USA) with a flow rate of 5 $\mu l\ min^{-1}$. The sample-cone voltage (V_c) was typically set to 40–45 V.

2.3. Spectroscopic determinations and data analysis

At all isolation and purification stages, the concentrations of HbA_1O_2 and $HbA_{1C}O_2$ were determined on a Shimadzu 2501 (Shimadzu Corp., Japan) UV–Vis spectrophotometer using a molar extinction coefficient of 13.8 $mM^{-1}\ cm^{-1}$ at 541 nm (Antonini & Brunori, 1971).

2.4. Crystallization

Crystallization was carried out using the hanging-drop vapour-diffusion method at 277 K. Prior to crystallization, the oxyhaemoglobin was concentrated to 20 $mg\ ml^{-1}$ in buffer containing 50 m*M* sodium/potassium phosphate pH 6.70. Prior to drop preparation, oxygen was bubbled through the haemoglobin and reservoir solutions. The oxygenation state of haemoglobin and the absence of

Table 1
Data-collection and processing statistics.

Values in parentheses are for the last resolution shell.

Space group	*C*2
Unit-cell parameters (Å, °)	a = 237.98, b = 59.27, c = 137.02, β = 125.4
Unit-cell volume (Å^3)	1563817
Asymmetric unit	$2 \times \alpha_2\beta_2 + \alpha\beta$
Solvent content (%)	49
Resolution range (Å)	15–2.3 (2.38–2.3)
No. of observations	204973
No. of unique reflections	64184
Completeness of data (%)	92.3 (71.0)
Merging R for all reflections (%)	3.4 (39.1)
Average $I/\sigma(I)$	23.7 (2.5)

methaemoglobin were controlled by spectrophotometric parameters (Soret band position, ratio D_{575}/D_{540}, absorption at 630 nm).

The standard oxyhaemoglobin crystallization method (Perutz, 1968) was adapted to the hanging-drop method. Several crystallization conditions were identified using PEG as a precipitant. These conditions were optimized by variation of pH, PEG molecular weight and protein concentration. The best quality crystals were obtained using a reservoir solution containing 100 m*M* sodium/potassium phosphate pH 6.70 and 25% PEG 4000. To prevent haemoglobin oxidation by heavy-metal ions, all reservoir solutions additionally contained 2 m*M* EDTA. 4 µl hanging drops containing 2 µl protein solution mixed with 2 µl reservoir solution were set up on siliconized cover slips over 0.5 ml reservoir solutions in 24-well plates. Single needle-like crystals with maximum dimensions of 0.1 × 0.2 × 2 mm (Fig. 2) grew at 277 K in approximately 2 d.

2.5. Data collection and analysis

Crystals were soaked in a cryoprotectant solution [reservoir solution with 25%(*v*/*v*) glycerol] for 10 s, picked up in a loop and then flash-cooled to 120 K in a stream of nitrogen gas prior to data collection.

Data sets were collected at the European Synchrotron Radiation Facility at the Swiss Light Source, Villigen, Switzerland.

Diffraction data were processed using the programs *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997; see Table 1 and Fig. 3).

3. Results

Gel analysis on the final fraction shows that only glycosylated haemoglobin tetramers are present in the purified sample.

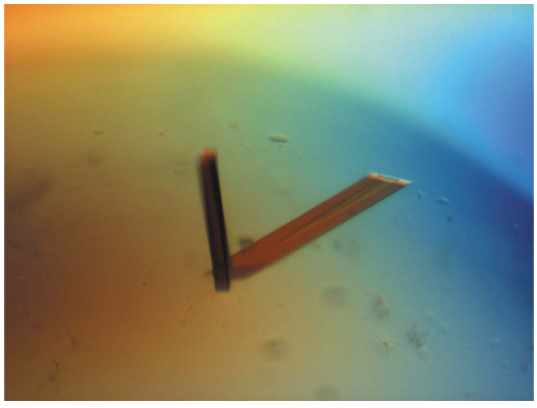

Figure 2
Crystal of human glycosylated haemoglobin.

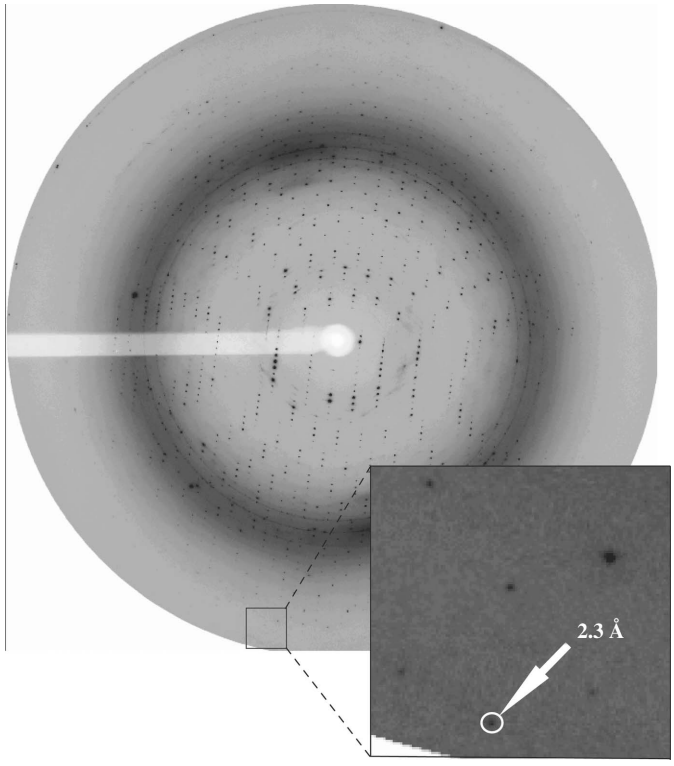

Figure 3
X-ray diffraction image of glycosylated haemoglobin. The inset shows diffraction spots at the resolution limit used in data processing.

Mass-spectrometric analysis of the final HbA_{1C} tetramers before and after crystallization showed the presence of modified haemoglobin (Fig. 1). In both cases, the mass spectrum shows the presence of glycosylated and non-glycolsylated α- and β-subunits, indicating the presence of tetramers containing one or two glycosylated α- and/or β-subunits.

The crystals belong to space group *C*2, with unit-cell parameters a = 237.98, b = 59.27, c = 137.02 Å, α = 90.00, β = 125.40, γ = 90.00°. The resolution limit is 2.3 Å.

Determination of the structure is being performed using molecular-replacement methods. In a first analysis, the solution shows 2.5 tetramers in the asymmetric unit. Preliminary analysis shows large conformational changes in the quaternary structure compared with the various models used. The process of rebuilding is under way.

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